Rhodopsin maturation defects induce photoreceptor death by apoptosis: a fly model for Rhodopsin\textsuperscript{Pro23His} human retinitis pigmentosa

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rhodopsin mutations result in autosomal dominant retinitis pigmentosa (ADRP), the most frequent being Proline-23 substitution by histidine (Rho\textsuperscript{P23H}). Although cellular and rodent animal models have been developed, the pathogenic mechanisms leading to Rho\textsuperscript{P23H}-induced cell death are still poorly understood. For this, we have used a Drosophila model by introducing a mutation in the fly rhodopsin-1 gene (Rh1\textsuperscript{P37H}) that corresponds to human Rho\textsuperscript{P23H}. Rh1\textsuperscript{P37H} transgenic flies show dominant photoreceptor degeneration that mimics age-, light-dependent and progressive ADRP. Moreover, we clarify the pathogenic mechanism of Rh1\textsuperscript{P37H} mutation that acts as an antimorph. First, we show the dual-localization of mutant Rhodopsin since most of Rh1\textsuperscript{P37H} accumulates in endoplasmic reticulum. Second, expression of mutant, mislocalized, Rhodopsin leads to cytotoxicity, via the activation of two stress-specific mitogen-activated protein kinases (MAPKs), p38 and JNK, which are known to control stress-induced apoptosis. In Rh1\textsuperscript{P37H} flies, visual loss and degeneration are indeed accompanied by apoptotic features and prevented by expression of p35 apoptosis inhibitor. Finally, we show for the first time that properly localized, mutant, Rhodopsin is active. Thus, the development of a fly model that faithfully reproduces the human disease sheds light onto the molecular defects causing ADRP thereby making it possible to devise potential therapeutic approaches.

INTRODUCTION

Retinitis pigmentosa (RP) represents a group of inherited disorders causing photoreceptor degeneration. Even though RPs are heterogeneous for disease severity and affected gene, they are all characterized by progressive blindness that is at present neither preventable nor curable (1). Twenty-five percent of autosomal dominant retinitis pigmentosa (ADRP) is linked to more than 100 different mutations scattered throughout the rhodopsin gene and affect different processes (http://www.sph.uth.tmc.edu/Retnet/) (2).

Proline substitution at position-23 by histidine (P23H) is the most frequent rhodopsin mutation causing ADRP (3). Moreover, Proline-23 by leucine (P23L) or by alanine (P23A) substitutions are also associated with ADRP (4,5).

Although transgenic mice and rats expressing Rho\textsuperscript{P23H} recapitulate dominant photoreceptor degeneration, the pathogenic mechanism is still under debate (6,7), for most of the other photoreceptor degenerative models (8). Moreover, controversial data on the subcellular localization of the mutant protein make it difficult to clarify the molecular bases of the Rho\textsuperscript{P23H}-induced degenerative phenotype. Drosophila has been successfully used to study human pathologies (9). Moreover, despite a different overall organization, fly and vertebrate retina share structural and molecular features: photoreceptor cells display similar structures sensitive to light, as Drosophila rhabdomeres are functionally equivalent to vertebrate photoreceptor outer-segments. Moreover, light stimuli are transduced by pathways that display many common key players (10,11).

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We have developed a transgenic approach to study RhoP23H-induced RP and shown that flies harboring a mutated Rhodopsin-1, Rh1P37H, analogous to human RhoP23H, develop dominant photoreceptor degeneration linked to progressive loss of vision. This age- and light-dependent phenotype recapitulates the phenotypes observed in RhoP23H patients and rodents. We show that mutant Rhodopsin is partially active and that it interferes with the phototransduction pathway. Although part of the Rh1P37H protein is properly transported to photoreceptor rhabdomeres, most of it is trapped in rough endoplasmic reticulum (ER), triggering activation of stress-specific p38 and JNK mitogen-activated protein kinases (MAPKs). Finally, the degeneration phenotype is rescued by p35 anti-apoptotic factor.

In conclusion, our fly model constitutes a promising genetic tool to uncover the molecular mechanisms underlying retinal degeneration and to identify potential therapeutic pathways.

RESULTS

Rh1P37H induces dominant light- and age-dependent retinal degeneration in flies

Drosophila compound eye is comprised 800 ommatidia, each harboring eight photoreceptors (R1–R8). Photoreceptor rhabdomeres are specialized portions of plasma membrane comprised thousands of microvilli corresponding to vertebrate photoreceptor outer-segments and containing phototransduction proteins.

Rhodopsin-1 (Rh1), which is expressed in outer photoreceptors (R1–R6), represents the major fly Rhodopsin and displays 22% amino acid identity with human Rhodopsin (12,13). Amino acid alignment of opsins throughout phyla highlights both conservation of Proline-23 in vertebrates and its correspondence with Proline-37 in Drosophila (http://www.gpcr.org/7tm/seq/001_004/001_004.MSF.mview2.html and Supplementary Material, Fig. S1). This residue lies in the N-terminal, extracellular part of the protein, a region of unknown function.

To develop a fly model for RhodopsinP23H (RhoP23H)-induced RP, we substituted Proline-37 by histidine and produced transgenic flies that, in addition to endogenous Rh1, express either Rh1P37H or wild-type Rh1 (Rh1 WT) under the control of the rh1 promoter (14) (referred to as Rh1P37H and Rh1 WT throughout the text).

RhoP23H patients and rodents present no vision deficit at birth (3,7,15,16). To determine whether Rh1P37H causes age-dependent retinal degeneration in flies, we investigated photoreceptor morphology as a function of age. Rhabdomeres of wild-type and transgenic (Rh1 WT and Rh1P37H) flies raised under day/night light conditions show no sign of photoreceptor degeneration at eclosion (Fig. 1A, C and E). Rh1P37H flies, however, display vacuoles in R1–R6 cytoplasm (data not shown) and slightly mislocalized (in 80% of the samples) (Fig. 1C). This very first degeneration signs are due to light exposure during pupal life, because pupae of the same genotype kept in the dark show no cytoplasm defect (data not shown).

Large number of R1–R6 photoreceptors degenerate by day 21 post-eclosion (Fig. 1D) and inner photoreceptors (R7 and R8) remaining intact (white arrow heads in Fig. 1D); in contrast, wild-type flies show no sign of degeneration throughout life (Fig. 1B). Morphological signs of degeneration such as reduced or absent rhabdomeres are observed by day 14 (Supplementary Material, Fig. S2A). This phenotype is
highly penetrant, because each ommatidium is affected (Fig. 1D). Like vertebrate RhoP23H, Rh1P37H induces a dominant phenotype, as flies carry both wild-type and mutant genes. Rh1WT-transgenic photoreceptors, in contrast, display normal morphology at day 21 (Fig. 1F).

RhoP23H-induced retinal degeneration is light-sensitive (17–20). Similarly, Rh1P37H flies reared in the dark do not display a degenerative phenotype, even after 35 days of post-eclosion (Supplemental Material, Fig. S2B). Furthermore, Rh1P37H-expressing photoreceptors (Fig. 1G and 1–K) were kept under constant light illumination during 24 h and subsequently analyzed, already present degenerative signs, such as involution of microvillar rhabdomere membranes (see arrows in Fig. 1I). Degenerating photoreceptor cytoplasm also contains a large number of lysosomes (see asterisks in Fig. 1G and J–K) and vacuoles (see ‘V’ in Fig. 1J and K), as found by electron microscopy. Such degenerative signs are not observed in control, Rh1WT-expressing, photoreceptors (Fig. 1H). Therefore, the fly Rh1P37H-induced dominant retinal degeneration is age- and light-dependent.

RhoP23H induces progressive blindness

RhoP23H-induced photoreceptor degeneration is associated with progressive blindness (6,15,16,21) and therefore we analyzed the visual activity of Rh1P37H flies at different ages.

Behavioral assay

Wild-type flies are attracted by light, a phenotype that is abolished in blind strains (22). We therefore assayed Rh1P37H flies for fast phototaxis, a behavioral assay measuring the visual activity of a fly population (23). In each test, flies are submitted to a sequence of five light stimuli in a countercurrent apparatus (Fig. 2A). Flies walking toward light are separated from those that do not, so that after the assay, the fly population is fractionated into walking toward light are separated from those that do not, resulting in a positive control (compare parts D and E with F and G of Fig. 3), showing that Rh1P37H ERG reduction is due to signaling impairment. One major and still open question concerning the RhoP23H-induced degeneration is whether the mutant protein is able to transduce the light signal correctly. To analyze the activity of Rh1P37H, we performed ERG in Rh1WT flies carrying the mutant or the wild-type transgene. Wild-type (WT) and Rh1WT flies display a significantly low PS (light shade columns in Fig. 2D). Finally, at day 1, both transgenic lines display a PS similar to that of wild-type flies and are therefore phototactic positive (solid coloured columns in Fig. 2D). Similar results are obtained in a genetic background that corresponds exactly to that of RP patients. Indeed, Rh1-null flies, in which one copy of mutated gene and one copy of WT gene have been reintroduced, show a PS of 4.7 at day 1 and 3.1 at day 14 (data not shown). Rh1P37H-induced defects are already detectable at day 7 and flies displaying intermediate visual activity (middle column of each genotype in Fig. 2D). Thus, Rh1P37H eye morphology correlates with visual behavior. Moreover, fast phototaxis provides a non-invasive, functional assay that detects progressive blindness well before morphological defects become manifest.

Electrophysiological assay

Phototactic behavior integrates parameters as diverse as locomotion, photoreceptor stimulation, phototransduction, synaptic transmission to second-order neurons and integration into the fly brain. To corroborate phototaxis data, we performed a direct measurement of visual activity by analyzing the electroretinogram (ERG). Fly ERG displays photoreceptor depolarization (Plateau), which corresponds to phototransduction cascade activation, and transient spikes following initiation and cessation of the light stimulus (ON and OFF), which results from synaptic activity (Fig. 2E) (25). This method allows for scoring of aged animals, which is not possible with the fast phototaxis assay, because locomotion decreases with age.

To accumulate further evidence of progressive blindness, we measured ERGs at different ages (days 1–56) in flies of the following genotypes: wild-type, Rh1WT, Rh1P37H, and Rh1-null (Fig. 2F). Rh1-null flies, which lack R1–R6 activity (12), provide a negative control and show reduced ERG resulting from R7 and R8 (Fig. 2E) (26). Rh1P37H flies display ERG amplitude similar to that of Rh1WT and WT flies at day 1 (Fig. 2E), confirming phototaxis data. With time, however, the Plateau amplitude of Rh1P37H flies gradually decreases compared with that of positive controls and by day 56, it reaches that of blind Rh1-null flies (Fig. 2F). Altogether, behavioral and electrophysiological data allow us to correlate morphological photoreceptor degeneration with progressive blindness.

Rh1P37H activates the visual signaling pathway

One major and still open question concerning the RhoP23H-induced degeneration is whether the mutant protein is able to transduce the light signal correctly. To analyze the activity of Rh1P37H, we performed ERG in Rh1-null flies carrying the mutant or the wild-type transgene. Wild-type (WT) and Rh1-null flies were used as positive and negative controls, respectively. The Plateau as well as the ON and OFF profiles indicate that both transgenes rescue the ERG phenotype of Rh1-null flies at day 1 (Fig. 3A and B). When compared with WT and Rh1WT flies ERG, however, Rh1P37H flies display significant reduced ERG amplitude (Fig. 3A and B). This is not due to altered photoreceptor morphology (compare parts D and E with F and G of Fig. 3), showing that Rh1P37H ERG reduction is due to signaling impairment.
Altogether, these data demonstrate that Rh1\textsuperscript{P37H} is able to transduce the light stimulus and that Rh1\textsuperscript{P37H} does not represent a null (complete loss of function) or a constitutively active (hypermorph) mutation. Finally, increasing the expression of the mutant protein does not improve the ERG amplitude (Fig. 3C), as revealed by using two copies of the mutant transgene. Thus, Rh1\textsuperscript{P37H} is not a hypomorph mutation (partial loss of function decreasing protein activity or expression).

**Rh1\textsuperscript{hsy-P37H} displays partial mislocalization in the fly eye**

The localization of the vertebrate mutant protein is matter of debate, because a large number of studies in cell cultures
assign a cytoplasmic localization (ER, agresome) to RhoP23H, whereas studies in vivo using transgenic animal models argue for RhoP23H proper localization to photoreceptor outer-segments (7,27–32). We therefore asked whether the degenerative phenotype is due to Rh1P37H misexpression/mislocalization. To specifically identify the mutant protein, we performed the analysis in flies lacking endogenous Rhodopsin by crossing Rh1P37H or Rh1WT transgenic flies with Rh1-null animals (referred to as Rh1P37H; Rh1-null and Rh1WT; Rh1-null, respectively). The amount of Rh1 expressed by Rh1P37H; Rh1-null and Rh1WT; Rh1-null flies is comparable, corresponding, respectively, to 53 and 52% of the Rh1 protein expressed in a wild-type strain, as quantified by densitometry (Fig. 4B).

Using anti-Rh1 immunolabeling in adult eyes, we found that Rhodopsin proteins colocalize with the rhabdomeric marker phalloidin, showing that Rh1P37H and Rh1WT are correctly targeted to R1–R6 apical microvilli (Fig. 4C and D), and are also expressed at proper developmental stage (70% of pupal life) (data not shown).

In conclusion, Rhodopsin labeling shows the same developmental profile, cell-specificity and subcellular localization for Rh1P37H and WT Rh1, consistent with the observation that Rh1P37H activates the phototransduction pathway.

One important question is to assess Rh1P37H subcellular localization in a more physiopathologic context, namely in the presence of wild-type Rh1, as it is the case in ADRP patients. Indeed, it is interesting to check whether both proteins were colocalized at the subcellular level, under degenerating conditions.

For this purpose, we generated a transgenic line that expresses an Rh1P37H protein tagged by hsv-epitope, under
the control of rh1 promoter (Rh1 \textsuperscript{hsv-P37H}). Hsv-epitope in fusion with C-terminus does not modify Rh1 properties (33). Moreover, similar to Rh1 \textsuperscript{P37H} flies, Rh1 \textsuperscript{hsv-P37H} flies display reduced PS at day 7 that reflects photoreceptor degeneration, which is in contrast to Rh1 \textsuperscript{hsv-WT}-expressing flies (data not shown). This tool allows us to specifically follow the behavior of the mutant protein in a genetic context that most faithfully reproduces that of patients affected by ADRP i.e., in presence of the endogenous wild-type Rhodopsin.

Rh1 \textsuperscript{hsv-P37H} and control Rh1 \textsuperscript{hsv-WT} flies were analyzed to compare subcellular localization of hsv-tagged Rhodopsins. Interestingly, anti-hsv labeling localizes to rhabdomeres in Rh1 \textsuperscript{hsv-WT} flies (Fig. 4F) (33), and also to both rhabdomeres (inset) and cytoplasm (arrows) in Rh1 \textsuperscript{hsv-P37H} flies (Fig. 4E). This reveals a dual Rh1 \textsuperscript{hsv-P37H} localization that is not detectable with anti-Rhodopsin-1 4C5 monoclonal antibody, as the latter labels only rhabdomeres (data not shown). Moreover, immunogold labelings analyzed by electron microscopy also show Rh1 \textsuperscript{hsv-P37H}, but not Rh1 \textsuperscript{hsv-WT}, localization to the ER (arrows, compare parts G and H of Fig. 4). Thus, Rh1 \textsuperscript{hsv-P37H} localizes both to rhabdomeres and ER, regardless of the presence of endogenous Rh1 (both in Rh1 \textsuperscript{WT} and Rh1 \textsuperscript{null} background) (data not shown).

Finally, we used a polyclonal anti-Rh1 antibody as a third method to reveal Rhodopsin (34) and formally demonstrated that mutant protein (with or without hsv tag) is localized both in rhabdomeres and cytoplasm, whereas wild-type protein only localizes at rhabdomeres (Fig. 4I and J and data not shown).

The hsv tool also allowed us to ask another important question concerning the possibility that the presence of mutant Rhodopsin affects the subcellular localization of wild-type endogenous Rh1. For this, we performed anti-hsv labeling on eye sections that express both Rh1 \textsuperscript{hsv-WT} and Rh1 \textsuperscript{P37H} transgenes in Rh1 null background. The fact that only wild-type protein is detected in rhabdomeres (Fig. 4K) clearly demonstrates that Rh1 \textsuperscript{P37H} mislocalization does not affect that of wild-type Rhodopsin.

Protein accumulation in ER has been shown to activate pathways that lead to phosphorylation and thereby activation of stress-specific MAPKs named p38 and JNK (35) which then translocate to the nucleus. The observed ER accumulation of Rh1 \textsuperscript{P37H} and the degenerative phenotype of R1–R6 prompted us to analyze the phosphorylation state of two such proteins in wild-type and mutant conditions.

**Figure 4.** Rh1 \textsuperscript{P37H} localizes to rhabdomeres and to ER. Transverse sections (A, C–K) of adult eyes (day 1), immunolabeled in green with anti-Rh1 (Rh1) (A, C, D), anti-hsv (hsv) (E, F), anti-Rh1 polyclonal antibody (I, J), respectively. Phalloidin, which labels rhabdomeric and sub-rhabdomeric (asterisks) F-Actin, in red. (A) Wild-type (WT); (C, D) (Rh1 \textsuperscript{P37H}; Rh1-null) and (Rh1 \textsuperscript{WT}; Rh1-null), respectively. Like endogenous Rh1 and Rh1 \textsuperscript{WT}, Rh1 \textsuperscript{P37H} accumulates in R1–R6 rhabdomeres delineated by dotted lines. (B) Anti-Rh1 western blot on head extracts: wild-type (WT), Rh1-null and WT or mutant transgenes into an Rh1-null background (Rh1 \textsuperscript{P37H}; Rh1-null) and (Rh1 \textsuperscript{WT}; Rh1-null). Same amount of total protein in each lane, b-tubulin as loading control (B). (E, G) Rh1 \textsuperscript{hsv-P37H}, Rh1 \textsuperscript{hsv-WT}, Rh1 \textsuperscript{WT}; (F, H) Rh1 \textsuperscript{hsv-P37H}; (I) Rh1 \textsuperscript{hsv-WT}, Rh1 \textsuperscript{P37H}; (G, H) Anti-hsv immunogold labeling analyzed by electron microscopy. Rhodopsin labeling at ER is indicated by black arrows. (I) (Rh1 \textsuperscript{hsv-P37H}, Rh1 \textsuperscript{WT}); (J) (Rh1 \textsuperscript{hsv-WT}, Rh1 \textsuperscript{WT}). In contrast with Rh1 \textsuperscript{WT} restricted in rhabdomes (J), anti-Rh1 polyclonal antibody confirms Rh1 \textsuperscript{P37H} dual localization in rhabdomeres and cytoplasm (arrows I). 1–7 indicate R1–R7 photoreceptors, respectively. Scale bars: 2 μm (A, C–E, I–K); 0.5 μm (G, H).
MAPKs in western blot assay from Rh1

expressing head extracts. The signal obtained using antibody against the phosphorylated form of p38 (activated p38) is stronger in Rh1

expressing head extracts (day 7 or 14) than in Rh1

head extracts (Fig. 5A). We obtained the same result by using Rh1

flies (data not shown). We also observed that phosphorylation of JNK protein, another stress-induced MAPK, is also increased in the mutant, albeit at lower level, as compared to p38 (Fig. 5B).

This clearly shows that Rh1

expression leads to stress-induced MAPK activation. Moreover, by looking at the subcellular level, we could assign this activation to the degenerating cells, as we specifically detected Phospho-p38 translocation in mutant photoreceptor nuclei (Fig. 5C and D).

**Blocking apoptosis rescues the Rh1

phenotype**

Previous studies realized on vertebrate models suggest that RhoP23H triggers an apoptotic pathway (19,36,37). To characterize the mechanisms of photoreceptor loss, we analyzed aged Rh1

ommatidia by electron microscopy and observed several apoptotic features such as dense nuclei (compare Fig. 6E-G), devolution of rhabdomic membranes and loss of sub-rhabdomeric structures (compare Fig. 6C, D and H and see Fig. 11). We also found that p35 anti-apoptotic protein (38) fully prevents the light-induced photoreceptor loss (Fig. 6B). Furthermore, p35 rescues rhabdomere and nucleus ultrastructure defects (Fig. 6D and F). Most importantly, p35 rescues the phototactic behavior (Fig. 6I). Indeed, Rh1

flies' PS at day 14 corresponds to 3.6, whereas that of Rh1

flies expressing p35 corresponds to 5.3 (Fig. 6I), which is very similar to the score of phototaxis-positive flies (see Fig. 2). Finally, p35 expression also partially rescues the ERG phenotype (Plateau amplitude: Rh1

= -5.84 mV; Rh1

+p35 = -8.04 mV) (Fig. 6J).

We therefore conclude that Rh1

-induced degenerative mechanisms converge onto an apoptotic pathway and that it is possible to prevent photoreceptor loss of function by blocking such pathway.

**DISCUSSION**

We here present a fly transgenic model for RhoP23H-induced RP that allows us to characterize the pathogenic mechanisms by functional and morphological assays. We show that mutant Rhodopsin is partially active and interferes with the phototransduction pathway. We also show that most of the mutant Rhodopsin is trapped in ER, which activates stress-MAPKs, and that the degeneration phenotype is rescued by p35 anti-apoptotic factor.

**The Rh1

fly: a model for RhoP23H-induced degeneration**

Rh1

induces age-dependent and progressive phenotypes. For patients and vertebrate models, young transgenic flies carrying mutant Rhodopsin display wild-type phototactic behavior, electrophysiological responses and photoreceptor morphology. Rh1

flies show first signs of blindness at day 7 post-eclosion (Fig. 2D). Moreover, in RhoP23H rodents (6,7,16,39), Rh1

flies show progressive functional (phototaxis and ERG) and morphological defects (Fig. 2D, data not shown, Supplementary Material, Fig. 2A). Finally, Rh1

induced degeneration is dominant and light-dependent, as seen in the vertebrate models (17-20).

These data clearly show that Rh1

transgenic flies faithfully reproduce the pathological events occurring in ADRP patients that carry the Rho

mutation. This allows us to use Drosophila as a novel animal model to study the RhoP23H-induced RP and to characterize the molecular mechanisms triggering degeneration.

**RhoP23H responds to light**

One of the most challenging questions is to determine the molecular pathways leading to photoreceptor degeneration. Depending on the rhodopsin mutation, distinct pathogenic mechanisms have been proposed, affecting protein activity or localization. Our data allow us to draw first conclusions on the cause of Rh1

-induced defects.
A frequently described cause of retinal degeneration involves Rhodopsin dysfunction: rhodopsin mutations failing to activate phototransduction (40) and mutations inducing light-independent constitutive activity lead to retinal degeneration in flies, transgenic mice and patients (41,42). Our electrophysiological data show for the first time that Rh1P37H is able to trigger photoreceptor depolarization and synaptic activity. This is the first evidence that the mutant protein is active, as the very early onset of degeneration of Rhodopsin transgenic mice lacking endogenous Rhodopsin prevents from evaluating the intrinsic activity of Rhodopsin (43). Moreover, although Rh1P37H displays a reduced ERG, it does not constitute a hypomorph mutation, as two copies of the mutant transgene in a genetic rhodopsin null background neither restore nor improve partially ERG wild-type amplitude. In addition, the fact that degeneration is not observed in flies kept in the dark speaks against Rh1P37H being a constitutively active mutation (Supplementary Material, Fig. S2B).

Our genetic data also allow us to exclude the possibility that Rh1P37H-induced degeneration is due to Rhodopsin excess, as transgenic Rh1WT expressed at comparable level as Rh1P37H does not induce degeneration. Therefore, Rh1P37H does not constitute a hypermorph mutation, which is consistent with the observation that expressing two doses of Rh1WT does not impair visual behavior (Supplementary Material, Table S1). By comparison, vertebrate RhoWT does trigger photoreceptor degeneration but only when overexpressed at extremely high levels (7). Finally, and more interestingly, performing the same dosage experiment with Rh1P37H further aggravates the mutant phenotype, as flies carrying one dose of Rh1P37H in a wild-type rhodopsin background see better than those carrying two doses of the same transgene (Supplementary Material, Table S1).

Altogether, the genetic approach allows us to demonstrate for the first time that Rh1P37H acts neither as a partial or complete loss of function (null/hypomorph) nor as a hypermorph or constitutively active mutation. Rather, Rh1P37H acts an antimorph, due to the cytotoxic effects induced by mislocalized Rh1P37H molecules (see subsequently). Thus, mutant, mislocalized, Rhodopsin indirectly interferes with the phototransduction cascade.

Rhodopsin P37H dual localization

A second cause of photoreceptor-dominant degeneration is abnormal Rhodopsin trafficking (44). Indeed, a large number of dominant mutations throughout Rhodopsin cause protein sequestration in ER in flies (45,46).

Concerning Rhodopsin P23H, subcellular localization of the mutant protein is still under debate. RhoP23H mislocalization has been observed in vertebrate transfected cells (27–31), whereas in transgenic animals it is properly targeted to photoreceptor outer-segments (7,28,32). The use of transgenic wild-type transgenic animals it is properly targeted to photoreceptor outer-segments (7,28,32). The use of transgenic wild-type and mutant-tagged Rhodopsins has made it possible to unambiguously address the issue of protein localization in vivo. Indeed, our immunolabeling data illustrate both localizations of dominant mutations throughout Rhodopsin (Supplementary Material, Table S1).

Altogether, the genetic approach allows us to demonstrate for the first time that Rhodopsin P37H acts neither as a partial or complete loss of function (null/hypomorph) nor as a hypermorph or constitutively active mutation. Rather, Rhodopsin P37H acts an antimorph, due to the cytotoxic effects induced by mislocalized Rhodopsin molecules (see subsequently). Thus, mutant, mislocalized, Rhodopsin indirectly interferes with the phototransduction cascade.
Rh1 monoclonal antibody is masked, either by misfolding or because altered protein interaction, as it has been described for the inactive form of Rh1 bound to Arrestin-2 (34). Most of the Rhodopsin-dominant mutations described in Drosophila, acting on mutant protein maturation trough ER, also interfere with the wild-type Rhodopsin transport (45,47). In the case of Rh1P37H, however, mutant Rhodopsin sequestration in ER does not interfere with the Rh1hsv-WT transport to rhabdomeres.

Finally, in contrast to many dominant rhodopsin mutations in Drosophila causing ER membrane accumulation (45,46), Rh1P37H flies show normal ER ultrastructure (Supplementary Material, Fig. S2C), similar to what observed in RhoP235H mice (7,28). Our data favor the hypothesis that Rhodopsin transport is partially impaired allowing a small proportion of Rh1P37H to properly localize to rhabdomeres, where it is functional.

Protein accumulation in ER has been described to trigger apoptosis in some neurodegenerative processes such as Alzheimer’s disease, through the so-called ER stress pathway (48). To respond to this ER stress and to get rid of protein excess, the unfolded protein response (UPR) signaling network is subsequently activated, which in turn finally leads to the activation of stress-MAPKs p38 and JNK (35). We have shown that P37H mutation triggers Rh1 sequestration in ER and triggers p38 and JNK activation, which is specific to photoreceptors.

Rh1P37H-induced degenerative mechanism leads to apoptosis

The fact that Rh1P37H photoreceptors undergo apoptosis and this phenotype is fully rescued by expressing p35 anti-apoptotic factor strongly suggest that Rh1P37H activation ultimately leads to caspase activation (49). Several mutations altering the phototransduction cascade (Rh1C200Y, rdgC, arr2 or norpA) inducing age- and light-dependent degeneration in flies are also fully (50–53) or partially (54) prevented by p35 expression (50–54). Most importantly, the observation that Rh1P37H-induced phenotype is morphologically and functionally rescued by p35 demonstrates a therapeutic effect of late-stage inhibition of apoptosis in adult flies. As apoptosis has been described in a variety of vertebrate models for retinal degeneration (55), the present data confirm the interference of apoptotic pathways as a promising therapeutic tool for human retinal dystrophies (56).

The activation of the UPR on ER stress has been described to trigger apoptosis, when such a pathway is not sufficient to get rid of the accumulated protein in ER (35). In view of our data, we propose that Rh1P37H accumulation in the ER causes apoptosis trough activation of a stress pathway that leads to p38 and JNK phosphorylation. In the future, it will be interesting to discuss the pathway leading to stress-MAPKs activation and to determine whether UPR is involved in it.

In conclusion, our data are emblematic of the conservation of the visual system between invertebrates and vertebrates. They validate the fly transgenic model to study the photoreceptor degeneration and shed light onto the pathogenic pathway associated with the Rh1P37H-induced RP. Use of flies makes it possible to perform genetic screens aiming at finding genes that modify Rh1P37H photoreceptor degeneration. This represents a promising avenue to identify viability factors that may be used for therapeutic purposes.

MATERIALS AND METHODS

Transgenesis and Drosophila lines

rh1 coding of 1747 bp and upstream sequences of 1000 bp are cloned in CasPer4. Eleven amino acid hsv-tag epitope coding sequences (bold) are added to rh1 3’ sequence (GCCAGCGAGGCSGAGTCCAAGGCACAAAAAGGACGACC CGAGGATCCCCAGGTAAAGGTTACCC). Transgenesis is performed in w1118 flies. Different lines obtained for each of the transgenes p(w+ rh1-Rh1WT) and p(w+ rh1-Rh1P37H) display similar phenotypes. Transgenic lines and wild-type control flies displaying same eye pigmentation are compared throughout the study. p(w+ rh1-Rh1WT), p(w+ rh1-Rh1P37H) and p(w+ rh1-Rh1hsv-37H) lines are referred to as Rh1WT, Rh1P37H and Rh1hsv-P37H, respectively. rh1-null allele is I17 (Bloomington stock center), and p(rh1+ rh1-Rh1WT) referred to as Rh1hsv-WT (33). p35 rescue experiments are performed using w,p(rh1-Gal4) and p(w+ rh1-Rh1P37H)p(w+ UAS-p35) flies (Bloomington stock center). Drosophila are raised on corn-medium, under day/nightlight conditions that correspond to 12 h dark/ light at 25 C. Constant illumination conditions are obtained by using two photosynthetic fluorescent tubes (in total 350 cd/m²) at 25°C. In all illumination conditions, pupae were reared in 12 h dark/light cycles.

Western blotting analysis

Heads are mashed in lysis buffer (20 mM phosphate buffer of pH 7, 1% SDS). One hundred and fifty micrograms of protein extracts are loaded on a 10% SDS–PAGE and transferred on nitrocellulose. Primary antibody is used for Western blotting analysis and blocked 1 h with 5% goat serum in PBST. Primary antibody is used for Western blotting analysis and blocked 1 h with 5% goat serum in PBST. Primary antibody is used for Western blotting analysis and blocked 1 h with 5% goat serum in PBST. Primary antibody is used for Western blotting analysis and blocked 1 h with 5% goat serum in PBST. Primary antibody is used for Western blotting analysis and blocked 1 h with 5% goat serum in PBST. Primary antibody is used for Western blotting analysis and blocked 1 h with 5% goat serum in PBST.
respectively. Rh1 expression is revealed by 4C5 mouse monoclonal antibody (1/50) (DSHB), anti-hsv monoclonal antibody (1/1000) (Novagen) or Rabbit polyclonal anti-Rh1 (1/500) (34) and F-Actin cytoskeleton by phalloidin coupled to TRITC (1/100) (Sigma). Slides are analyzed with Leica DMRE confocal microscope.

For immunogold electron microscopy, heads are dissected and fixed overnight at 4°C in 4% PFA, 0.1% glutaraldehyde in Na phosphate buffer (0.1 M, pH 7.4), dehydrated in a graded ethanol series and embedded in araldite-epon. Ultrathin sections of 70 nm collected on nickel grids are blocked with 1% NGS diluted in 0.01 m PBS–0.5% Tween 20, incubated 3 h at room temperature with anti-hsv (1/2000), washed with BSA (0.2%) and incubated 2 h with anti-mouse antibody conjugated to 10-nm colloidal gold particles (Aurion) (1/20). After post-fixation in 2.5% glutaraldehyde, sections are contrasted 15 min with 5% uranyl acetate and examined under a CM 12 Philips electron microscope.

Fast phototaxis

Countercurrent apparatus with six tubes of length 15 cm is placed horizontally, light source is switched on and flies allowed to walk during 30 s toward a photosynthetic fluorescent tube (700 cd/m²). Tubes are shifted in order to reiterate the test five times. Results are presented on histograms as fly percentage in each tube or as PS (ΣNi)/ΣN, where N is the number of flies in the ith tube.

Electroretinogram

Cold-anesthetized flies are immobilized in clay. A tungsten electrode (0.5–1 MΩ, Intracell) is inserted in the back of the head and a glass electrode filled with 3 M KCl (2–6 MΩ) is poked through the cornea. Flies are dark-adapted for 2 min before recording. A white LED (1 cd, 60° light beam, Radiospares) is located at 1.5 cm from the head. The flash intensity (700 cd/m²) is chosen as the minimal intensity that consistently produces maximal ERG Plateaus. Signals are filtered at 2 kHz and digitized at 10 kHz, using a MultiClamp 700 A amplifier, Digidata 1322 A interface and pClamp-8 software (Axon Instruments). Flash intensity and duration are controlled through pClamp and Digidata.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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